IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Jia-He Li and Jie Zhang	Examiner:	Wang, Shengjun
Serial No.:	09/182,645	Group Art Unit:	1617
Filed:	October 30, 1998	Docket No.:	60014.0001US01
Title:	Pharmaceutical Compositions Containing Poly(ADP-Ribose) Glycohydrolase Inhibitors and Methods of Using Same		

CERTIFICATE UNDER 37 C.F.R. 1.10
'Express Mail' mailing label number: EL 900437864 US
Date of Deposit: May 10, 2002
I hereby certify that this paper or fee is being deposited with the United States Postal Service 'Express Mail Post Office To Addressee' service under 37 C.F.R 1.10 and is addressed: Box AF, Commissioner for Patents, Washington, D.C. 20231.

By: Shanda Clemmons
Name: Shanda Clemmons

APPELLANTS' BRIEF UNDER 37 C.F.R. § 1.192

BOX AF
Commissioner for Patents
Washington, D.C. 20231

Dear Commissioner:

A Notice of Appeal was filed in this application on March 1, 2002. This Brief, filed in triplicate, is filed with the requisite fees and Appendix.

I. Real Party in Interest

The real party in interest is Guilford Pharmaceuticals, Inc., the assignee of the present application.

II. Related Appeals and Interferences

The assignee, the assignee's legal representatives, and the appellants are unaware of any other appeals or interferences that will affect, be directly affected by, or have a bearing on the Board's decision in this appeal.

III. Status of Claims

Claims 46-49 are pending in the present application. Original claims 1-25 have been withdrawn with traverse from consideration as being drawn to a nonelected invention. Original claims 28-31, and 35-38 have been withdrawn with traverse as being drawn to a nonelected species. Claims 26-27, 32-34, and 39-45 have been canceled.

IV. Status of Amendments

No amendments were filed after the Final Office Action.

V. Summary of Invention

The claimed invention is directed to a method for treating neural or cardiac tissue damage resulting from a disease or condition by administrating to a mammal in need thereof a therapeutically effective amount of an inhibitor of poly(ADP-ribose) glycohydrolase (known as "PARG"). (Specification, pg. 25, lines 10-25; pg. 32, lines 2-8, 19-24; pg. 68, lines 18-24; pg. 69, line 7 through pg. 70, line 7; pg. 71 lines 8-19; pg. 72 line 20 through pg. 73, line 17; claim 46.) The inventors have discovered that PARG inhibitors can be used to inhibit or decrease free radical induced cellular energy depletion, cell damage, or cell death, thereby allowing the

treatment of a disease or condition resulting from cell damage or death due to necrosis or apoptosis. (See, e.g., Specification, pg. 31, lines 6-10.)

In one embodiment of the invention, PARG inhibitors were discovered to treat cardiovascular tissue damage resulting from cardiac ischemia, reperfusion injury, cardiovascular disease, heart attack, and vascular stroke. (Specification, pg. 72 line 72 through pg. 73, line 17; pgs. 86-87, Example 2; pgs. 89-90, Examples 5-7.) In another embodiment, PARG inhibitors are used to treat neural tissue damage in a mammal resulting from cerebral ischemia, neurodegenerative disease, neurological disease, and head trauma. (Specification, pg. 73, lines 17-23; pgs. 84-86, Example 1.)

VI. Issues Presented on Appeal

The issues presented for this appeal are (1) whether claims 46-49 directed to a method of administrating PARG inhibitors to treat neural and cardiac tissue damage resulting from certain diseases and conditions are anticipated by Wang's sugar free ginseng tea (Patent no. 1077644A),[1] Ning's coffee-flavored ginseng tea (Patent no. 1113711A),[2] and Tanuma's AB (JP 3-205402)[3] or AC (JP 4-13684)[4] cancer treating PARG inhibiting lignin glycoside; and (2) whether claims 46-49 are rendered obvious by Wang or Ning in view of Tanuma AB and AC.

---

[1] A true and accurate translation of Wang is attached as Exhibit ("Ex.") A.
[2] A true and accurate translation of Ning is attached as Exhibit B.
[3] A true and accurate translation of Tanuma AB is attached as Exhibit C.
[4] A true and accurate translation of Tanuma AC is attached as Exhibit D.

VII. Grouping of Claims

Claims 46-47 should be considered as a group.

Claims 48-49 should be considered as a group.

VIII. Argument

Appellants' claims are to methods of treating neural or cardiac tissue damage resulting from a disease or condition by administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of poly(ADP-ribose) glycohydrolase ("PARG"). The Wang and Ning references cited by the Examiner in the Final Office Action do not disclose or suggest a *therapeutic treatment* for any disease, let alone for diabetes or ischemia. Neither do they disclose or suggest the other claim limitations, i.e., treating neural or cardiac tissue damage with a PARG inhibitor. When read in combination with Wang or Ning, the Tanuma references do not solve the noted deficiencies. The rejections of claims 46-49 should be reversed.

A. Claims 46-49 are not Anticipated by Wang, Ning, or Tanuma

In the Final Office Action dated December 3, 2001 ("Action"), claims 46-49 were rejected under 35 U.S.C. § 102(b) as being anticipated by Wang, Ning, and Tanuma (AB and AC). Specifically, the Examiner asserted that (1) Wang teaches a method of treatment of diabetes comprising administering ginseng to the patient, and (2) Ning teaches a method of treatment of ischemia comprising administering ginseng to the patient. The Examiner also noted that Tanuma teaches that ginseng hot water extract contains "the lignin glycoside herein. Therefore the claimed method herein read on the method taught by Wang and Ning." (Action, pgs. 2-3.)

Applicants respectfully submit that the Examiner has erroneously interpreted the Wang and Ning references. With respect to Wang, the Examiner reads this reference to disclose a treatment for diabetes. This is factually incorrect. Wang teaches a procedure for the production of a sugar free tea product. Because the tea product is sugar free, the consumption of such tea product is "suitable for diabetes patients." (Wang, Ex. A, pg. 1.) The sugar free tea product is suitable for diabetes patients not because it acts as a therapeutic that treats the underlying disease of diabetes, but rather because the product does not worsen an already known disease or condition. As Wang states "[t]he advantage of said product is that it contains no sugar and is suitable for ingestion by all people." (Wang, Ex. A, pgs. 1, 3).[5] The sugar free tea product disclosed in Wang is not a therapeutic agent that treats diabetes or any other disease or condition. Thus at least two elements of claim 46—therapeutic treatment of neural or cardiac tissue damage resulting from a disease or condition in a mammal—are plainly not described in Wang. The Examiner's rejection with respect to Wang is improper.

The Ning reference shares certain key disqualifying traits with Wang in that it describes "a nourishing health beverage of coffee-flavored ginseng tea." (Ning, Ex. B, pg. 1.) Similar to Wang, Ning extols the unsupported and mysterious "health protecting effects" of coffee-flavored ginseng tea. But again like Wang, Ning does not disclose the elements of claim 46, i.e., therapeutically treating neural or cardiac tissue damage resulting from a disease or condition. Rather, Ning teaches the following about its health beverage:

> This formula utilizes ginseng which supplements energy and promotes production of body fluid, stimulates central nerve system,

---

[5] Wang also mentions, without one word of explanation, that its sugar free tea product has "therapeutic and preventing effects." (Wang, Ex. A, pgs. 1, 3.) Such a generic, undefined, and unsupported claim does not provide any guidance to one of ordinary skill in the art who might consider the therapeutic usefulness of sugar free ginseng tea.

> reduces blood sugar and improves heart contraction and heart rate . . . .
>
> It can be used as a concentrate or beverage, combining medicine, health protecting effects and beverage in one and suitably applicable to middle-aged and elderly individuals experiencing prostration due to a long illness, neurasthenia, myocardial ischemia and cerebral and physical exhaustion.

(Ning, Ex. B, pgs. 1-2.)

Whatever one of skill in the art believes about these unsupported protective health effects of Ning's coffee-flavored ginseng tea, they do not describe a therapeutic treatment of neural or cardiac tissue damage resulting from a disease or condition as claimed in claims 46-49. Ning only teaches that a person who drinks coffee-flavored ginseng tea may temporarily receive certain body stimulating health effects. Coffee-flavored ginseng tea is not treating or curing myocardial ischemia or another disease or condition. Accordingly, the Examiner's anticipation rejection based on Ning should be reversed. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 231 U.S.P.Q. 81 (Fed. Cir. 1986) ("It is axiomatic that for prior art to anticipate under 102 it has to meet every element of the claimed invention.").

Applicants also submit that a PARG inhibitor is not disclosed in Wang or Ning, at least one that is sufficiently described to allow one of skill in the art to think it acts in a therapeutic manner with respect to damaged tissue. The Examiner incorrectly assumes that the ginseng described in Wang and Ning must necessarily contain lignin glycoside (a known PARG inhibitor disclosed in the Tanuma references) and that such lignin glycoside must be present in a therapeutically effective amount. (Action, pg. 3.) Neither Wang nor Ning mention lignin glycoside or any other PARG inhibitor. The Examiner has simply read lignin glycoside into these references because he inherently assumes it must be present in the ginseng. That is a faulty assumption.

There are at least dozens of distinct compounds/agents in any one type of ginseng, and there are many different types of ginseng. Moreover, there is no consensus among those of ordinary skill in the art as to which particular component of ginseng may have a health benefit—if indeed ginseng has any particular health benefit whatsoever. The Examiner's unwarranted reading of this claim limitation (i.e., a PARG inhibitor) into Wang and Ning should be disregarded.

Moreover, the Examiner's contention that "Tanuma teach that ginseng hot water extract contain[s] the lignin glycoside" and therefore reads on the method taught by Wang and Ning is fraught with highly relevant factual inaccuracies. (Action, pg. 3.) Wang, Ning, and Tanuma (both Tanuma AB and AC describe identical procedures for this particular point) teach three entirely different extraction procedures involving ginseng.

The first point to note is that Tanuma does indeed teach that a hot or boiling water extraction is necessary to properly extract the lignin glycoside.[6] (Tanuma AC, Ex. D, pgs. 5-7.) But that is only the first extraction step. Left completely unmentioned by the Examiner is that the boiling water extract is then further extracted with a basic aqueous solution, which is subsequently acidified and diluted with ethanol, whereby a ginseng precipitate is recovered and used as the active agent. (Tanuma AC, Ex. D, pg. 5.) Therefore, if one takes a whole ginseng root, and follows the detailed extraction procedures in Tanuma, lignin glycoside is recovered in presumably relatively pure form for possible therapeutic use.

Wang, on the other hand, describes a procedure wherein only 30% of a ginseng root (head and tail portion only) is extracted with water and concentrated. (Wang, Ex. A., pgs. 2-3.) Contrary to Tanuma, the extraction is not done with boiling water. Wang's non-boiling water

---

[6] The only example in Tanuma AB and AC describes the hot water extraction of pine cones—not ginseng—to ultimately provide the desired lignin glycoside. (Tanuma AC, Ex. D, pgs. 6-7.)

extraction is also not followed up with a further basic extraction (and subsequent isolation procedures). Given these extraction discrepancies, there is no reasonably possibility that one of ordinary skill in the art would read Wang to inherently include the lignin glycoside (at least in a therapeutic amount) that is described in Tanuma.

Ning discloses yet another ginseng extraction that is different than Tanuma. Ning tells one of skill in the art to boil (in water) and concentrate 6-16 parts ginseng, 60-100 parts Acanthopanax root or Acanthopanax bark or Acanthopanax, and 10-90 parts pilose antler blood or pilose antler extract.[7] Whereas Tanuma conducts a series of extractions to a boiled extract of 100% ginseng root to provide a relatively pure lignin glycoside PARG inhibitor, Ning simply concentrates (after boiling) this rather exquisite concoction to leave what the Examiner surprisingly assumes is pure lignin glycoside. Obviously the amount of ginseng extract (and to an unknown smaller proportion the purported lignin glycoside) is a small part of this concoction—certainly not of the amount (if any at all) necessary to eventually yield a therapeutically effective concentration of lignin glycoside to be administered as directed in claims 46-49. The Examiner's assumption that the lignin glycoside disclosed in Tanuma is inherently present in Wang and Ning represents clear error and must be disregarded with respect to the anticipatory rejection.

With respect to the Tanuma references, it is not clear from the entirety of the Examiner's comments in the Final Office Action whether either Tanuma reference is being cited as an anticipatory reference or solely used to support his reading of lignin glycoside into Wang and Ning. Both Tanuma references disclose lignin glycoside as a PARG inhibitor. Tanuma AB

[7] Twenty to thirty parts of wolfberry fruit and 600-1000 parts of sugar and a suitable amount of citric acid are added to the aforementioned concentrated extract.

teaches that inhibitory PARG activity is useful for treatment and prevention of malignant tumor (anti-cancer) and viral infection. (Tanuma AB, Ex. C, pgs. 4-5.) Tanuma AC discloses that lignin glycoside used as a PARG inhibitor is useful as an anti-cancer and anti-viral agent, a cytokine intensifying agent, and a cytokine production inducing agent. (Tanuma AC, Ex. D, pgs. 4-5.)

Neither Tanuma reference, however, discloses or suggests using a PARG inhibitor to treat neural or cardiac tissue damage resulting from a disease or condition as in claim 46. And neither Tanuma reference discloses or suggests using a PARG inhibitor to treat neural or cardiac tissue damage resulting ischemia, reperfusion injury, neurodegenerative disease, neurological disease, head trauma, cardiovascular disease, heart attack, and vascular stroke as in claim 47. The Examiner's view is apparently consistent with applicants' position as he has previously noted: "Tanuma do[es] not specifically teach employment of the lignin glycoside for treating disease[s] directly related to the activity of poly(ADP-ribose) polymerase, e.g., cellular energy depletion, apoptosis or neurological disorder." (May 31, 2001 Office Action, Paper no. 22, pg. 4.)

In the Final Office Action the applicants' attention was also directed to In re Swinehart, which was cited to support the Examiner's comment "that mode of action elucidation does not impart patentable moment to otherwise old and obvious subject matter." Applicants believe the Examiner is focusing on the wrong part of the claims for novelty. Claims 46-49 are not claiming the old "thing" (e.g., lignin glycoside) referred to in In re Swinehart on the basis of newly discovered properties or functions. Rather, applicants are claiming (see, e.g., claim 46) PARG inhibitors that are used in novel methods of treating neural or cardiac tissue damage resulting from a disease or condition. Claims 47-49 exemplify a particular embodiment of the invention

by expressly naming preferred diseases and conditions. Such method claims are well recognized as patentable subject matter. 35 U.S.C. § 100(b); In re Schoenwald, 964 F.2d 1122, 22 U.S.P.Q.2d 1671 (Fed. Cir. 1992); Loctite Corp. v. Ultraseal Ltd., 781 F.2d 861, 875, 228 U.S.P.Q. 90, 99 (Fed. Cir. 1985) ("Even if a composition is old, a process using a known composition in a new and unobvious way may be patentable.").

B. The Claims are Patentable Over Wang or Ning in View of Tanuma AB or AC

Claims 46-49 were also rejected under 35 U.S.C. § 103(a) as being unpatentable over Wang or Ning in view of Tanuma (AB and AC). (Action, pgs. 3-5.) The Examiner's obviousness rejection has no foundation because (1) there is no objective motivation to combine Wang or Ning with Tanuma, and (2) as primary references, Wang and Ning do not teach or suggest any of the claim elements and their combination with Tanuma leaves one of skill in the art only with the deficient teachings of Tanuma with respect to claims 46-49.

When an obviousness rejection relies on the combination of prior art references, there must be some objective evidence to combine the references to yield the claimed invention. *In re Dembiczak*, 50 U.S.P.Q.2d 1614, 1618 (Fed. Cir. 1999); *see also In re Fritch*, 23 U.S.P.Q.2d 1780, 1783 (Fed. Cir. 1992) (holding the examiner can satisfy the burden of obviousness in light of combination "only by showing some objective teaching [leading to the combination]"). Accordingly, the Federal Circuit has made clear that it is imperative that the prior art references must teach or suggest the combination of the references to yield the claimed invention. That standard for a viable obviousness rejection has not been and cannot be satisfied here.

There would have been no motivation for one skilled in the art to combine Wang or Ning with Tanuma (AB or AC) to yield the claimed invention. Starting with the primary references (Wang and Ting), as noted earlier, these references teach a health beverage containing either

sugar free or coffee-flavored ginseng tea. Contrary to the Examiner's unfounded conclusions, the consumption of such ginseng teas provides no plausible therapeutic treatment of any underlying health disease or condition. Neither Wang nor Ning teach or suggest a PARG inhibitor of any type. There is also no mention or suggestion of treating neural or cardiac tissue damage in the references. The primary references of Wang and Ning simply do not teach or suggest any of the claim limitations of claims 46-49.

The secondary references cited by the Examiner, Tanuma AB and AC, disclose that PARG inhibitors exist (e.g., lignin glycoside) and are therapeutically useful in mammals as anti-tumor and anti-viral agents. When the references are viewed together, one of ordinary skill in the art is not going to logically expand upon Tanuma's limited therapeutic uses of PARG inhibitors with the teachings of Wang and Ning. There was and still is no objective connection between Tanuma and the Wang and Ning references, even with the impermissible hindsight of applicants' invention. *See C.R. Bard, Inc. v. M3Sys., Inc.*, 48 U.S.P.Q.2d 225, 1232 (Fed. Cir. 1998) (describing "teaching or suggestion or motivation [to combine]" as an "essential evidentiary component of an obviousness holding.").

Even assuming, *arguendo* that the references are properly combined, there is no teaching or suggestion that a PARG inhibitor (e.g., Tanuma references) can be used to treat neural or cardiac tissue damage resulting from a disease or condition in a mammal in need thereof. *See* claim 46. Neither Wang nor Ning plausibly teach or suggest an actual therapeutic treatment of any underlying disease. Thus, while the Tanuma references teach PARG inhibitors, they do not cure the deficiencies of Wang and Ning by disclosing or suggesting the methods of use described in claims 46-49. Indeed, the Examiner has already noted that Tanuma only teaches certain uses of lignin glycoside—uses not directed to the claimed method of treating neural or cardiac tissue

damage resulting from a disease or condition, such as ischemia and reperfusion injury. (See Action, pgs. 3-4).

Applicants respectfully request that the Board reverse this obviousness rejection.

C. Separately Patentable Claims

Claims 48-49 are directed to particular diseases or conditions that a PARG inhibitor will treat if administered to a mammal in need thereof. These claims represent a species of the more generic claims 46-47. As such, applicants believe claims 48-49 are separately patentable from claims 46-47.

IX. Conclusion

For the foregoing reasons, the rejections of Claims 46-49 should be reversed.

Respectfully submitted,

Mark J. Pino
Reg. No. 43,858
202.625.8377

Date: May 10, 2002

Merchant & Gould P.C.
3200 IDS Center
80 South Eighth Street
Minneapolis, MN 55402-2215

APPENDIX

46. A method of treating neural or cardiac tissue damage resulting from a disease or condition in a mammal in need thereof, comprising administering to said mammal a therapeutically effective amount of an inhibitor of poly(ADP-ribose) glycohydrolase.

47. The method of claim 46, wherein the disease or condition is ischemia, reperfusion injury, neurodegenerative disease, neurological disease, head trauma, cardiovascular disease, heart attack, and vascular stroke.

48. The method of claim 47, wherein the disease or condition is ischemia.

49. The method of claim 47, wherein the disease or condition is reperfusion injury.

Exhibit A

Chinese Patent No. 1077644A

PEOPLE'S REPUBLIC OF CHINA PATENT OFFICE
INVENTION PATENT PUBLICATION JOURNAL
PUBLICATION NO. 1077644A

Int. Cl^5:	A 61 K 35/78
Filing No.:	93102286.X
Filing Date:	March 8, 1993
Publication Date:	October 27, 1993
Number of pages of explanation:	2
BJ NO.:	1456

A GINSENG TEA BAG AND PRODUCTION METHOD THEREOF

Applicant:	Shawan Health Product Plant, Fanyu City Shashi Public Road, Shawan County, Fanyu City, Guangdong Province 511483
Inventor:	Zhi-Qiang Wang
Patent Agency:	Zhongshang Medical University Patent Office
Representative:	Mingxing Chen

Abstract

This invention pertains to a ginseng tea bag and production method thereof. It is characterized in that the ingredient of the composition of said product comprises western ginseng granulates prepared by a special process using original root of American ginseng. The advantage of said product is that it contains no sugar and is suitable for ingestion by all people including males and females, young and old. It is particularly suitable for diabetes patients, with therapeutic and preventing effects, while it has the characteristics of being convenient to carry, easy to ingest and easy in the control of doses.

Claims

1. A ginseng tea bag, characterized in that the ingredient of the composition comprises American ginseng granulates, which are packed in tea bags as ginseng tea bags.

2. A production procedure for ginseng tea bags, characterized by:

(1) Taking a given quantity of original root of American ginseng, washing away the dirt on the skin, using 70% of the American ginseng root for steam-vapor sterilization in a closed system, drying and pulverizing into particles and sieving to obtain a given amount of qualified granulates (A), while keeping the remaining portion as a powder (B);

(2) Carrying out water extraction on the 30% head and tail portion of the American ginseng and concentrating to give concentrate (C);

(3) Drying the American ginseng extracted with water and pulverizing to give a powder and granulates (D);

(4) Combining B, C and D obtained above to prepare granulates, and mixing them with phase A after drying, and packing in multiple films to give ginseng tea bags.

Explanation

This invention pertains to a ginseng tea bag and production method thereof.

American ginseng products currently commercialized on the market include: original ginseng root and ginseng chip, granular water-down agent [unconfirmed translation]. The granular water-down agent contains sugar, making it unsuitable for some people (for example, diabetes patients) to ingest. As for the original ginseng root and ginseng chip, though not containing sugar, their ingestion is not convenient for an average person because the dose is not easily controllable, making it difficult to achieve a therapeutic and preventing effect for diseases. Side effects could even occur when there is an overdose.

The objective of the present invention lies in providing a ginseng tea bag that can be easily carried, in which the dose is easily controllable and water-down ingestion is convenient, and a production method thereof.

The present invention is described in detail below using an application example.

The ingredients of the composition of the present invention are American ginseng granulates, which are packed in tea bags as ginseng tea bags. The production procedure comprises: Taking a given quantity of original root of American ginseng, washing away the dirt on the skin, using 70% of the American ginseng root for steam-vapor sterilization in a closed system, drying and pulverizing into particles and sieving to obtain a given amount of qualified granulates (A), while keeping the remaining portion as powder (B); carrying out water extraction on the 30% head and tail portion of the American ginseng and concentrating to give concentrate (C) and drying the American ginseng extracted with water and pulverizing it to give a powder

and granulates (D); combining B, C and D obtained above to prepare granulates, and mixing them with phase A after drying, and packing into multiple films to give ginseng tea bags.

Application example

Take 1000 g original American ginseng root, wash away the dirt on the skin and use 700 g of the American ginseng root for steam-vapor sterilization in a closed system, dry and pulverize into particles and sieve to obtain a given amount of qualified granulates (A), while keeping the remaining portion as powder (B); carry out water extraction on the 300 g head and tail portion of the American ginseng and concentrate it to give concentrate (C) and dry the American ginseng extracted with water and pulverize it to give powder and granulates (D); combine B, C and D obtained above to prepare granulates, and mix them with phase A after drying, and pack into small bags to give ginseng tea bags containing 1.8 g American ginseng granulates.

The above application example is not to be construed as limiting the present invention.

The advantage of the present invention is that the content has no sugar and is suitable for ingestion by all people including males and females, young and old. It is particularly suitable for diabetes patients, with therapeutic and preventing effects. Additionally, said product is convenient to carry, easy to ingest with easy control of the doses.

B

Chinese Patent No. 1113711A

PATENT OFFICE OF THE PEOPLE'S REPUBLIC OF CHINA
PUBLIC DESCRIPTION OF INVENTION PATENT
PATENTS NO. 1113711A

Int. Cl^6.:	A 23 L 1/29 A 23 L 2/38 A 61 K 35/78
Filing No.:	94110348.X
Filing Date:	June 24, 1994
Publication Date:	December 27, 1995
BJ No.:	1456

COFFEE-FLAVORED GINSENG TEA AND PRODUCTION METHOD THEREOF

Applicant:	Dai-De Ning 10-2-7 10th Building, Yanjiang Xiao Qu, Tonghua City, Jilin Province 134001
Inventor:	Shi-De Ning
Patent Agency:	Tonghua City Patent Office
Representatives:	Wei Wang, Jianming Wang

Abstract

This invention discloses a nourishing health beverage of coffee-flavored ginseng tea, comprising ginseng, Acanthopanax root, pilose antler, wolfberry fruit, sugar and a suitable amount of citric acid; it can be used as a concentrate or beverage, combining medicine, health protecting effects and beverage in one and suitably applicable to middle-aged and elderly individuals experiencing prostration due to a long illness, neurasthenia, myocardial ischemia and cerebral and physical exhaustion.

Claims

1. A nourishing health coffee-flavored ginseng tea, characterized in that it comprises 6-16 parts ginseng extract, 60-100 parts Acanthopanax root or Acanthopanax bark or Acanthopanax

extract, 10-90 parts pilose antler blood or pilose antler extract, 20-30 parts wolfberry fruit, 600-1000 parts sugar and a suitable amount of citric acid.

2. A production method of the coffee-flavored ginseng tea according to the description of Claim 1, characterized by:

(1) Ginseng, Acanthopanax root or Acanthopanax bark or acanthopanax core, and pilose antler are cooked in water respectively and concentrated to prepare concentrated extracts, followed by drying, pulverizing and dividing into parts;

(2) A given part of wolfberry fruit is cooked, filtered, and the boiled liquid is mixed with a given part of sugar, followed by drying and pulverizing, or a given part of wolfberry fruit is dried, pulverized and mixed with a given part of sugar;

(3) The aforementioned pulverized parts and contents are mixed until homogeneous, and a suitable amount of citric acid is added to obtain coffee-flavored ginseng tea.

This invention pertains to a nourishing health coffee-flavored ginseng beverage and the production method thereof.

The prior art consists of ginseng tea as a nourishing health beverage, which is prepared directly from ginseng by pulverizing it and adding sugar. The drawback of the tea is that it has one simple function with a narrow health goal, and the oral sensation of the ginseng powder from direct pulverization is far from satisfactory.

The objective of the present invention lies in providing a nourishing health coffee-flavored ginseng beverage, without adding coffee yet having the taste of coffee, and the production method thereof.

The objective of the present invention is achieved in the following manner: The coffee-flavored ginseng tea comprises 6-16 parts ginseng extract, 60-100 parts Acanthopanax root or Acanthopanax bark or Acanthopanax extract, 10-90 parts pilose antler blood or pilose antler extract, 20-30 parts wolfberry fruit, 600-1000 parts sugar and a suitable amount of citric acid. This formula utilizes ginseng which supplements energy and promotes production of body fluid, stimulates central nerve system, reduces blood sugar and improves heart contraction and heart rate, aided by Acanthopanax which eliminates wind-dampness syndrome and strengthens muscle and bone, while the two combined together provide the effect of regulating and restoring the flow of *qi* and producing body fluid and tranquilizing; it is assisted with pilose antler blood or pilose antler extract which strengthens muscle and bone, helps produce vital essence, activates blood and eliminates toxic substances, and aided by wolfberry fruit which helps normalize the functions of the liver and kidney, nourishing blood and clarifying the eyes. This formula comprises key and supplementary ingredients having a synergetic effect, and is able to provide nourishment and energy for weakness and fatigue, mental fatigue and neurasthenia, to strengthen muscle and bone, produce body fluid and tranquilize, and strengthen the kidney and spleen and

rejuvenate body fluid and blood, while providing effects of eliminating night sweats, spermatorrhoea, back and leg pain, loss of appetite, insomnia and overdreaming as well as of eliminating wind-dampness syndrome. Acanthopanax root and ginseng taste like coffee after being cooked in water and extracted. Acanthopanax root can be substituted by Acanthopanax bark or Acanthopanax core or plants of the Acanthopanax family.

The production method of the coffee-flavored ginseng tea comprises cooking ginseng, Acanthopanax root or Acanthopanax bark or Acanthopanax extract, and pilose antler in water respectively and concentrating to prepare concentrated extracts, followed by drying, pulverizing and dividing into parts, and cooking a given part of wolfberry fruit, filtering, and mixing the boiled liquid with a given part of sugar, followed by drying, pulverizing, or drying a given part of wolfberry fruit, pulverizing and mixing with a given part of sugar, and finally mixing the aforementioned pulverized components and contents (parts) until homogeneous, followed by adding a suitable amount of citric acid to obtain coffee-flavored ginseng tea. The product can be prepared in powder form, granulate form, cube form or as a beverage, and is suitable for ingestion by middle-aged and elderly people with long-term illnesses and prostration, neurasthenia, myocardial ischemia, weak kidneys cerebral and physical exhaustion.

Ingestion method: Each bag contains 15 g, which is poured into 250 mL hot water and ingested.

The advantages of the present invention are: 1. It combines medicine, health protection and beverage in one product; 2. It has a coffee taste without using coffee; 3. It has a wide range of nourishing value and health protecting effects.

Application Example 1

Pulverize 6 kg ginseng extract, 60 kg Acanthopanax root extract, 10 kg pilose antler extract, 20 kg wolfberry fruit, 600 kg sugar and a suitable amount of citric acid, mix until homogeneous and package into bags.

Application Example 2

Mix 12 kg ginseng extract, 80 kg Acanthopanax root, 60 kg pilose antler extract, 25 kg wolfberry fruit, 800 kg sugar and a suitable amount of citric acid until homogeneous, pulverize and package into bags.

Application Example 3

Pulverize 16 kg ginseng extract, 100 kg Acanthopanax root extract, 90 kg pilose antler extract, 30 kg wolfberry fruit, 1000 kg sugar and a suitable amount of citric acid, mix until homogeneous and package into bags.

(19)Japanese Patent Office
(11)Patent Application Laid Open
(12)**Published Unexamined Patent Application 3-205402(1991)**

(13)Int.Cl.[5]	identification mark	reference number
C 08 B 37/00	Q	7624-4C
A 61 K 31/725	ADU	
	AED	7431-4C

no request for examination 5 claims (7 pages in total)

(54)Title of the Invention

Lignin glycoside and its use

(21)Patent Application 2-113048(1990)
(22)Application Date April 28.1990

(23)Inventor Seiti Tanuma
1-10 Omon-cho, Hachioji-si, Tokyo
(71)Applicant The Green Cross Corp.
1-3-3 Imabasi,Tyuou-ku,Osaka-si,Osaka
(74)Attorney Patent attorney Hajime Takashima

SPECIFICATION

1. Title of the Invention

Lignin glycoside and its use

2. What is claimed is:

1. A lignin glycoside having the following properties.

(i) Lignin and polysaccharide are bonded.

(ii) The molecular weight is 60000 to 140000.

(iii) The bonding ratio of lignin and polysaccharide is 1:1 to 20:1 (molecular ratio).

(iv) Polysaccharide is composed of 60 to 70% of uronic acid, and 30 to 40% of neutral sugar.

2. A poly-(ADP-ribose) glycohydrolase inhibitor mainly composed of lignin glycoside.

3. A cytokine action intensifying agent mainly composed

of lignin glycoside.

4. A cytokine production inducing agent mainly composed of lignin glycoside.

5. A cancer immunotherapeutic agent mainly composed of lignin glycoside.

3. Detailed Description of the Invention

[Industrial Field of Utilization]

The present invention relates to a novel lignin glycoside and its use.

[Prior Art · Problems to be Solved by the Invention]

Almost all existing anticancer drugs have the action of suppressing DNA synthesis or cell division, but present similar actions also to normal cells. By making use of a slight difference that the cancer cells are fast in cell division while normal cells are slow, treatment is established by giving more damages to cancer cells. Damages received by normal cells are expressed as side effects, and it is an important point in cancer treatment how far the body can withstand such side effects.

By nature, cancer treatment should be based on biology and biochemistry of cancer cells, but actually such cancer therapy is not realized yet.

As causes of cancer, traditionally, carcinogen, radiation and virus have been pointed out, and it has been clarified that cells are turned cancerous by the gene information of the cancer virus, and the term "oncogene" has been coined. It was later hypothesized that the oncogene is present also in normal cells and is switched on by some cause to make the cells cancerous. As the hypothesis was discussed and proved further, and the hypothesis is generally accepted nowadays.

In genomes of higher animals, there are 50 kinds or more proto-oncogenes that can be oncogenes, and they play important physiological functions in proliferation and differentiation of normal cells. Therefore it gives rise to possibility of control of cell proliferation or control of cancer at the level

of genes or at the level of gene products. It is an object of the invention to develop a cancer remedy capable of suppressing the stage of expression of oncogene by a specific inhibitor. Using mouse mammary tumor cells of which expression of inserted mouse mammary tumor virus (MMTV) is controlled by corticoid, it has been discovered that expression of MMTV gene is triggered by de-poly-ADP-ribose reaction in chromatin protein. That is, as poly-ADP-ribose is decomposed, the local change of chromatic structure is considered to be finally related to promotion of bonding and transfer of RNA polymerase to promoter [Journal of Biological Chemistry, 258: 15371 (1983)].

Accordingly, the present inventor expected that the oncogene would not be activated if decomposition of poly-ADP-ribose could be inhibited, and hence isolated and refined poly-(ADP-ribose) glycohydrolase which is an enzyme responsible for decomposition of ADP-ribose from the human placenta, and searched for compounds having an inhibitory action on this enzyme, and then discovered a potent inhibitory activity in certain natural compounds.

Moreover, such compounds were found to have completely new activities for intensifying the cell killing action and cell division inducing action possessed by the tumor necrosis factor (TNF), and this intensifying effect was discovered to be derived from heightening of bonding affinity of TNF to the TNF receptor. Still more, the compounds were known to have an action of inducing production of TNF and interleukin I from macrophage. By further promoting the investigation, a novel compound usable as a pharmaceutical having an anticancer action based on poly-(ADP-ribose) glycohydrolase inhibition, and TNF and other cytokine induction and their action intensifying effect was discovered, and the invention was completed.

[Means of Solving the Problems]

That is, the invention is characterized by the following.

1. A lignin glycoside having the following properties.

(i) Lignin and polysaccharide are bonded.

(ii) The molecular weight is 60000 to 140000.

(iii) The bonding ratio of lignin and polysaccharide is 1:1 to 20:1 (molecular ratio).

(iv) Polysaccharide is composed of 60 to 70% of uronic acid, and 30 to 40% of neutral sugar.

2. A poly-(ADP-ribose) glycohydrolase inhibitor mainly composed of lignin glycoside.

3. A cytokine, that is, tumor necrosis factor (TNF) action intensifying agent mainly composed of lignin glycoside.

4. A cytokine (TNF, IL-1) production inducing agent mainly composed of lignin glycoside.

5. A cancer immunotherapeutic agent (by combined use with TNF or other cytokine) mainly composed of lignin glycoside.

The lignin glycoside of the invention is a bonded composition of lignin and sugar (polysaccharide). Lignin and sugar (polysaccharide) are bonded by ether bonding. The bonding ratio of lignin: constituent sugar is, for example, about 1: 3 to 5 by weight. The molecular ratio of lignin and polysaccharide is, for example, about 1 to 20: 1.

The sugar component of lignin glycoside is composed of uronic acid and neutral sugar. The composition is, for example, about 60 to 70% of uronic acid, and 30 to 40% of neutral sugar.

The neutral sugar includes glucose, galactose, mannose and arabinose. The composition is, for example, about 15 to 20 mol % of glucose, 25 to 30 mol % of galactose, 35 to 50 mol % of mannose, and 10 to 15 mol % of arabinose.

These constituent sugars have a structure of saccharides on the whole, and form a polysaccharide.

The molecular weight of lignin glycoside is about 80000 to 140000, and the molecular weight of polysaccharide portion is about 40000 to 100000. The molecular weight of lignin portion is about 1000 to 10000.

The lignin glycoside of the invention is composed of the following elements: for example, about 35 to 45 wt.% of C atom, 1 to 10 wt.% of H atom, and 45 to 64 wt.% of O atom.

The lignin glycoside of the invention is prepared in the

following manner.

The starting material includes, for example, tea (leaves, twigs), lithospermum root, trisaccharide root, ginseng, pine (cones, leaves), grass dogwood (stem).

The material is treated in the solvent (for example, hot water, ethanol, acetone). The treating time is about 1 to 15 hours. The treated material is extracted in an alkaline solution (0.1 to 1N sodium hydroxide, ammonium, etc.). The extracted liquid is adjusted to pH 4 to 6, and ethanol is added by 1 to 5 times in amount, and the precipitation fraction is recovered. The precipitation fraction is refined by gel filtration, and the active portion is recovered.

Thus obtained lignin glycoside can be treated by dialysis, centrifugal separation, freeze-drying, etc.

The lignin glycoside of the invention has poly-(ADP-ribose) glycohydrolase inhibitory action, and TNF production induction and intensifying effect of its action, and presents poly-(ADP-ribose) glycohydrolase inhibitory activity, and TNF production induction and intensifying activity of its action to mammals including humans (human, horse, dog, mouse, guinea pig, rat, etc.), and is useful for treatment and prevention of malignant tumor and viral infection as poly-(ADP-ribose) glycohydrolase inhibitor, and TNF production induction and its action intensifying active agent.

The lignin glycoside of the invention is administered either orally or parenterally.

The lignin glycoside is administered either alone or in a form of pharmaceutical preparation together with a pharmaceutically allowable carrier. The preparation is manufactured by a known method. The dosage forms include tablet, capsule, powder, suppository, injection, etc.

The lignin glycoside is administered, for example, by oral route, usually by about 0.1 to 100 mg/kg of body weight a day either once or in several divided portions, but the dose may be changed depending on the age, body weight and/or severity of the disease to be treated and reaction to treatment.

Toxicity test

The toxicity of the lignin glycoside of the invention in mice was investigated, and, by oral administration, the LD_{50} value was 100 mg/kg or more, and the LD_{50} value was extremely high, and this is a compound with a broad safety region.

[Embodiments]

Embodiment 1

The lignin glycoside was extracted in the following operation.

Example

Pinecone

↓

Extraction in hot water

The boiling time varies with the amount of pinecones or

↓ amount of water, but is usually 2 hours x 3 times.

Extraction in ethanol

Pinecones extracted in hot water are half dried, and immersed in ethanol, and let stand overnight at room

↓ temperature.

Extraction in acetone

Pinecones extracted in ethanol are half dried, and immersed in acetone, and let stand overnight at room

↓ temperature.

Extraction in 0.3N sodium hydroxide (or ammonia) solution

Pinecones extracted in acetone are dried by lamp, and extracted in 0.3N sodium hydroxide solution while stirring for 6 hours (or overnight). Acetic acid is added to this extracted liquid, and the pH is returned to 5.0. The

↓ precipitate is removed by high speed centrifugal operation.

Precipitation in ethanol

An equivalent amount of ethanol is added to the extracted liquid, and let stand overnight in a cold room. The precipitate is collected by high speed centrifugal operation.

↓ The precipitate is dissolved in water and dialyzed in water.

Freeze-drying

The dialyzed solution is freeze-dried, and powder is ↓ obtained.

Gel filtration

The freeze-dried powder is refined by Sepharose CL-4B (the moving bed is 0.1 N NaOH). Active fractions are collected, acetic acid is added to return the pH to 5.0, and an equivalent amount of ethanol is added, and letting stand in ice for 1 to 2 hours, the precipitate is collected by high speed centrifugal operation. The precipitate is dissolved in 10% ethanol, and is further refined by Toyopearl HW-40F (the moving bed is 10% ethanol). Active fractions are collected, dialyzed in water, and freeze-dried, and powder is obtained.

Embodiment 2

To investigate the characteristic of the structure of sugar portion (glycone) and non-sugar portion (aglycone) of the lignin glycoside, the glycoside was separated into glycone and aglycone by methanolysis (decomposition into methanol and hydrochloric acid) or chlorite ($NaClO_2$) method, and analyzed. The results are as follows.

[Analysis of glycone]

Molecular weight: 60000 to 100000 by Sepharose CL-4B gel filtration method.

Composition of sugar a)	(%)	(total wt.%)
Uronic acid	63.4	48.8
Neutral sugar	36.6	28.2

It is a feature that 2/3 of glycone is Uronic acid.

Composition of neutral sugar b)	(mol %)
Glucose	18.2
Galactose	27.4
Mannose	40.2

Arabinose	13.8
Fucose	0

Neutral sugar contains glucose, galactose, mannose, and arabinose, but does not contain fucose.

a) Uronic acid was determined by carbazole method, and neutral sugar by phenol sulfate method.

b) The composition of neutral sugar was analyzed by gas chromatography after transforming methyl glycoside produced by methanolysis into trimethyl.

[Analysis of aglycone]

Molecular weight: 4000±2000 by Sepharose CL-6B gel filtration method.

Analysis of infrared absorption (IR)

IR was measured by using KBr disk.

As a result, an absorption having a peak at 3400 cm^{-1} was detected in a range of 3500 to 3700 cm^{-1}. This absorption indicates the presence of phenolic hydroxyl group. The absorption around 1600 cm^{-1} shows an aromatic double bond. Since there is no absorption of carbonyl group around 1700 cm^{-1}, there is no ester bond as noted in tannin, and it is proved to be a compound polymerized by ether bond as observed in lignin.

Including the fingerprint region, the entire spectrum is extremely similar to that of lignin (alkali).

From the above results of IR spectrum, the aglycone of this glycoside is estimated to be a lignin-like compound, not tannin-like compound.

Analysis of ultraviolet absorption (UV)

Maximum absorption was detected at 280 nm, and minimum absorption at 260 nm.

280/260 = 1.02

Lignin also has a similar UV spectrum.

280/260 = 1.03

The presence of aromatic group (probably phenol) is indicated by UV spectrum.

Analysis of electron spin resonance (ESR)

Same as in lignin, g = 2.004 ESR signal is detected, and it is known to contain a structure having a stable free radical. Such signal is not observed in tannin.

[Analysis of lignin glycoside]

The feature as the entire lignin glycoside is as follows.

Element analysis	(weight %)
C	39.83
H	4.41
O	55.73
N	0.03 or less
S	0

Since nitrogen and sulfur are not contained, it is free from protein and sulfate group, and the molecular weight by Sepharose CL-4B gel filtration method is about 110000.

The bonding ratio of lignin and sugar is about 1:4 by weight.

Therefore, it is a feature of this glycoside that it contains uronic acid by about 50% of the total weight as sugar component (glycone). The neutral sugar includes glucose, galactose, mannose, and arabinose. The non-sugar component (aglycone) is composed of lignin.

This glycoside is an O-glycoside having the reduction end lactol hydroxyl group of sugar bonded in ether form with alcoholic or phenolic hydroxyl group of lignin by dehydration and condensation. The bonding ratio of lignin and sugar is about 1:4 by weight, and in particular it is a lignin glycoside (as classified by the feature of aglycone) with molecular weight of about 110000 with a large content of uronic acid of acid sugar.

The estimated structural model of the glycoside is as shown in the diagram.

Test example 1

Inhibitory effect on poly-(ADP-ribose) glycohydrolase

To a buffer for assay (0.01% bovine serum albumin, 10 mM mercaptoethanol, 50 mM potassium phosphate, pH 7.0), ^{3}H-(ADP-ribose)$_{n=13}$ was added, and to 27 µl thereof, further, the substance to be tested and nuclear derivative poly-(ADP-ribose)

glycohydrolase solution prepared from human placenta were added to make up 30 μl in total, which was incubated for 1 hour at 37°C. Later, the reaction solution was absorbed in DE81 filter paper, and the filter paper was washed in water, ethanol and acetone, and was dried, and the unreacted substrate ^{3}H-(ADP-ribose) was measured by liquid scintillation counter, and the inhibitory action of the test substance on this enzyme was investigated. Results are shown in Table 1, which shows all tested substances inhibited poly-(ADP-ribose) glycohydrolase dose-dependently.

Table 1

Inhibitory activity of lignin glycoside on poly-(ADP-ribose) glycohydrolase

Concentration of lignin glycoside (μg/ml)	Activity of poly-(ADP-ribose)glycohydrolase (%)
0	100
1	83
3	48
10	17
30	8

Test example 2

Inhibitory effect on gene expression

The gene expression system used in the test was 341 strains of mouse mammary tumor cells having mouse mammary tumor virus (MMTV) genes which are saccharide corticoid susceptible genes. The cell, in the presence of saccharide corticoid, expresses 35S RNA, and 24S RNA further undergoing splicing. This expression can be detected by using cDNA of the env portion. Herein, the test substance was added to 341 strains by 30 μg/ml, and incubated for 30 minutes at 37°C, and dexamethazone was added to the system by 10^{-7}M, and it was further incubated for 1 hour. Then, 341 cells were collected, high molecular RNA was extracted by guanidine-hydrochloric acid method, and after treating for 5 minutes at 60°C (20 mM MDPS, pH 7.0, 5 mM sodium sulfate, 1

mM EDTA), electrophoresis was conducted (40 V, 16 h) by 1.2% agarose gel (same buffer). Consequently, transferring to nitrocellulose, ^{32}P-MMTV-DNA (cDNA specific to env) was hybridized, and an autoradiogram was prepared by X-ray film. Using the autoradiogram, the concentration of 35S and 24S RNA bands was measured by densitometer, and the RNA expression amount was determined, and the result was compared with the control without addition of test substance, and the RNA expression inhibitory rate was calculated. As the result is shown in Table 2, the test substance presented the MMTV gene expression inhibitory action.

Table 2

Action of lignin glycoside on expression of mouse mammary tumor virus (MMTV) gene

	Dexamethazone (10^{-7}M)	Lignin glycoside (30 μ g/ml)	Sensitivity of 35S, 24S bands
1	+	-	++++
2	-	+	+
3	-	-	+
4	+	+	++

1: positive control, 2 and 3: negative control, 4: test group

Test example 3

Carcinostatic effect on mouse experimental tumor

In the abdominal cavity of mouse, 1 x 10^6 tumor cells of sarcoma 180 were transplanted, and the test substance was continuously administered intraperitoneally for 1 to 4 days after transplantation. The antitumor activity was determined by the survival rate by comparison with the normal saline administration group. The experiment was terminated on the 45th day after tumor transplantation. The results are shown in Table 3, and the test substance presented a carcinostatic action.

Table 3

Carcinostatic action of lignin glycoside on mouse tumor sarcoma

Sample	Dose (mg/kg)	T/C (%)
None		100
Lignin glycoside	40×4	108
	20×4	206
	10×4	120
	5×4	91

The lignin glycoside was administered for 4 days consecutively from the day after transplantation.

Test example 4

Intensifying effect of TNF action on mouse fibroblastoma cell L-929

The cell killing action of TNF can be evaluated by measuring the stain concentration by staining the surviving viable cells by making use of adhesion of L-929 cells to Petri dish. Accordingly, the test substance was added to L-929 cells, actinomycin D was added by 4 μg/ml, and further TNF was added to incubate for 18 hours at 37°C. The plate was washed in normal saline, and dead cells were removed. Then viable cells were stained with 0.1% crystal violet, the stained viable cells were dissolved in 0.5% SDS, the pigment concentration was measured at OD 590 nm, and the intensifying effect on TNF action was studied. The results are shown in Table 4, in which all test substances intensified the cell killing action of TNF dose-dependently.

Table 4

Intensifying effect of TNF action on mouse fibroblastoma cell L-929

Lignin glycoside concentration (μg/ml)	CD_{50} concentration of TNF(g/ml)	Intensifying factor(%)
0	0.034	100

3	0.015	221
10	0.0062	548
30	0.0044	773
100	0.0074	458

Test example 5

Carcinostatic effect by combined use of TNF on mouse experimental tumor

By transplanting 1 x 10^6 cells of sarcoma 180 tumor subcutaneously in mice, the test substance and TNF were consecutively administered for 1 to 5 days after transplantation. The antitumor activity was determined by the survival rate by comparison with saline administration group. The experiment was terminated on 45th day after transplantation. The results are shown in Table 5, and the test substance presented a potent carcinostatic effect by concomitant use of TNF.

Table 5

Effect of concomitant use of lignin glycoside and TNF on mouse tumor sarcoma 180

Sample	Concentration (mg/kg)	T/C (%)
0		100
TNF		118
TNF+	40×4	191
Lignin glycoside	20×4	298
	10×4	254
	5×4	188

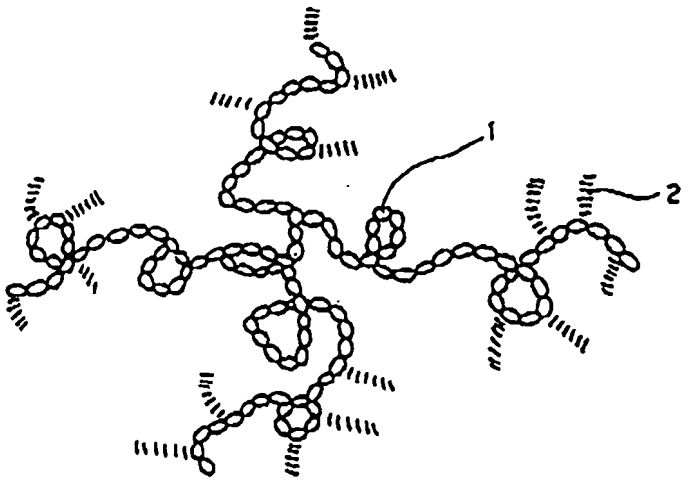
Lignin glycoside was administered, together with TNF, for 4 days consecutively from the day after transplantation.

4. Brief Description of the Drawing

The drawing shows the estimated structure of the lignin glycoside of the invention.

1: Sugar
2: Lignin

Applicant: The Green Cross Corp.
Attorney: Hajime Takashima, patent attorney



(19)Japanese Patent Office
(11)Patent Application Laid Open
(12)**Published Unexamined Patent Application** **4-13684(1992)**

(51)Int.Cl.[5]	identification mark	reference number
C 07 G 1/100		8318-4H
A 61 K 31/715	ADU	9164-4C
C 07 G 3/00		8318-4H
C 08 B 37/00	Q	7624-4C
C 12 N 9/99		

(43)Laid Open January 17, 1992
no request for examination 2 claims (6 pages in total)

(54)Title of the Invention
Lignin glycoside and its use

(21)Application No. 2-113049(1990)
(22)Application Date April 28.1990

(23)Inventor Seiti Tanuma
1-10 Omon-cho, Hachioji-si, Tokyo
(71)Applicant The Green Cross Corp.
1-3-3 Imabasi,Tyuou-ku,Osaka-si,Osaka
(74)Attorney Patent attorney Hajime Takashima

SPECIFICATION

1. Title of the Invention
Lignin glycoside and its use

2. What is claimed is:
1. A lignin glycoside having the following properties.
(i) Lignin and polysaccharide are bonded.
(ii) The molecular weight is 8000 to 10000.
(iii) The bonding ratio of lignin and polysaccharide is 1:1 to 2:1 (molecular ratio).
(iv) Polysaccharide is composed of 10 to 20% of uronic acid, and 80 to 90% of neutral sugar.

2. A poly-(ADP-ribose) glycohydrolase inhibitor mainly composed of lignin glycoside.

3. Detailed Description of the Invention

[Industrial Field of Utilization]

The present invention relates to a novel lignin glycoside and its use.

[Prior Art · Problems to be Solved by the Invention]

Almost all existing anticancer drugs have the action of suppressing DNA synthesis or cell division, but these drugs present similar actions also to normal cells. By making use of a slight difference that the cancer cells are fast in cell division while normal cells are slow, treatment is conducted by giving more damages to cancer cells. Damages received by normal cells are expressed as side effects, and it is an important point in cancer treatment to what extent the body can withstand such side effects.

By nature, cancer treatment should be based on biology and biochemistry of cancer cells, but actually such cancer therapy is not realized yet.

As causes of cancer, traditionally, carcinogen, radiation and virus have been pointed out, and it has been clarified that cells are turned cancerous by the gene information of the cancer virus, and the term "oncogene" has been coined. It was later hypothesized that the oncogene is present also in normal cells and is switched on by some cause to make the cells cancerous. As the hypothesis was discussed and proved, and is generally accepted nowadays.

In genomes of higher animals, there are 50 kinds or more proto-oncogenes that can be oncogenes, and they play important physiological functions in proliferation and differentiation of normal cells. Therefore it gives rise to possibility of control of cell proliferation or control of cancer at the level of genes or at the level of gene products. It is an object of the invention to develop a cancer remedy capable of suppressing the stage of expression of oncogene by a specific inhibitor.

Using mouse mammary tumor cells of which expression of inserted mouse mammary tumor virus (MMTV) is controlled by corticoid, it has been discovered that expression of MMTV gene is triggered by de-poly-ADP-ribose reaction in chromatin protein. That is, as poly-ADP-ribose is decomposed, the local change of chromatic structure is considered to be finally related to promotion of bonding and transfer of RNA polymerase to promoter [Journal of Biological Chemistry, 258: 15371 (1983)].

Accordingly, the present inventor expected that the oncogene would not be activated if decomposition of poly-ADP-ribose could be inhibited, and hence isolated and refined poly-(ADP-ribose) glycohydrolase which is an enzyme responsible for decomposition of ADP-ribose from the human placenta, and searched for compounds having an inhibitory action on this enzyme, and then discovered a potent inhibitory activity in certain natural compounds.

Further promoting the investigation, a novel compound usable as a pharmaceutical having an anticancer action based on poly-(ADP-ribose) glycohydrolase inhibition was discovered, and the invention was completed.

[Means of Solving the Problems]

That is, the invention is characterized by the following.

1. A lignin glycoside having the following properties.

(i) Lignin and polysaccharide are bonded.

(ii) The molecular weight is 8000 to 10000.

(iii) The bonding ratio of lignin and polysaccharide is 1:1 to 2:1 (molecular ratio).

(iv) Polysaccharide is composed of 10 to 20% of uronic acid, and 80 to 90% of neutral sugar.

2. A poly-(ADP-ribose) glycohydrolase inhibitor mainly composed of lignin glycoside.

The lignin glycoside of the invention is a bonded composition of lignin and sugar (polysaccharide). Lignin and sugar (polysaccharide) are bonded by ether bonding. The bonding ratio of lignin: constituent sugar is, for example, about 1 to 2: 1 by weight. The molecular ratio of lignin and

polysaccharide is, for example, about 1 to 2: 1.

The sugar component of lignin glycoside is composed of uronic acid and neutral sugar. The composition is, for example, about 10 to 20% of uronic acid, and 80 to 90% of neutral sugar.

The neutral sugar includes glucose, galactose, mannose and arabinose. The composition is, for example, about 25 to 30 mol % of glucose, 40 to 45 mol % of galactose, 20 to 25 mol % of mannose, and 5 to 10 mol % of arabinose.

These constituent sugars have a structure of saccharides on the whole, and form a polysaccharide.

The molecular weight of lignin glycoside is about 8000 to 10000, and the molecular weight of polysaccharide portion is about 2000 to 4000. The molecular weight of lignin portion is about 4000±20000.

The lignin glycoside of the invention is composed of the following elements: for example, about 35 to 45 wt.% of C atom, 1 to 10 wt.% of H atom, and 50 to 55 wt.% of O atom.

The lignin glycoside of the invention is prepared in the following manner.

The starting material includes, for example, pinecone, tea (leaves), grass dogwood, trisaccharide root, and etc.

The material is treated in the solvent (for example, hot water, ethanol, acetone). The treating time is about 1 to 15 hours. The treated material is extracted in an alkaline solution (0.1 to 1N sodium hydroxide, ammonium, etc.). The extracted liquid is adjusted to pH 4 to 6, and an equivalent amount of ethanol is added, and the supernatant fraction is recovered. The supernatant fraction is refined by gel filtration, and the active portion is recovered.

Thus obtained lignin glycoside can be treated by dialysis, centrifugal separation, freeze-drying, etc.

The lignin glycoside of the invention has poly-(ADP-ribose) glycohydrolase inhibitory action, and presents poly-(ADP-ribose) glycohydrolase inhibitory activity to mammals including humans (human, horse, dog, mouse, guinea pig, rat, etc.), and is useful for treatment and prevention of

malignant tumor and viral infection as poly-(ADP-ribose) glycohydrolase inhibitor.

The lignin glycoside of the invention is administered either orally or parenterally.

The lignin glycoside is administered either alone or in a form of pharmaceutical preparation together with a pharmaceutically allowable carrier. The preparation is manufactured by a known method. The dosage forms include tablet, capsule, powder, suppository, injection, etc.

The lignin glycoside is administered, for example, by oral route, usually by about 0.1 to 100 mg/kg of body weight a day either once or in several divided portions, but the dose may be changed depending on the age, body weight and/or severity of the disease to be treated and reaction to treatment.

Toxicity test

The toxicity of the lignin glycoside of the invention in mice was investigated, and, by oral administration, the LD_{50} value was 100 mg/kg or more, and the LD_{50} value was extremely high, and this is a compound with a broad safety region.

[Embodiments]

Embodiment 1

The lignin glycoside was extracted in the following operation.

Example

Pinecone

↓

Extraction in hot water

The boiling time varies with the amount of pinecones

↓ or amount of water, but is usually 2 hours x 3 times.

Extraction in ethanol

Pinecones extracted in hot water are half dried, and immersed in ethanol, and let stand overnight at room

↓ temperature.

Extraction in acetone

Pinecones extracted in ethanol are half dried, and

immersed in acetone, and let stand overnight at room
↓ temperature.

Extraction in 1N sodium hydroxide (or ammonia) solution

Pinecones extracted in acetone are dried by lamp, and extracted in 1N sodium hydroxide solution while stirring for 6 hours (or overnight). Acetic acid is added to this extracted solution, and the pH is returned to 5.0. The precipitate is removed by high speed centrifugal
↓ operation.

Precipitation in ethanol

An equivalent amount of ethanol is added to the extracted solution, and let stand overnight in a cold room. The precipitate is removed by high speed centrifugal operation,
↓ and the supernatant is dialyzed in water.

Freeze-drying

The dialyzed solution is freeze-dried, and powder is
↓ obtained.

Gel filtration

The freeze-dried powder is refined by Sepharose CL- 4B (the moving bed is 0.1 N NaOH). Active fractions are collected and dialyzed in water, and freeze-dried, and powder is obtained. This freeze-dried powder is dissolved in 10% ethanol, and is further refined by Toyopearl HW-40F (the moving bed is 10% ethanol). Active fractions are collected, dialyzed in water, and freeze-dried, and powder is obtained.

Embodiment 2

To investigate the characteristic of the structure of sugar portion (glycone) and non-sugar portion (aglycone) of the lignin glycoside, the glycoside was separated into glycone and aglycone by methanolysis (decomposition into methanol and hydrochloric acid) or chlorite ($NaClO_2$) method, and analyzed. The results are as follows.

[Analysis of glycone]

Composition of sugar [a]	(%)	(total wt.%)
Uronic acid	1.4	15.1
Neutral sugar	8.9	84.9

It is a feature that 5/6 of glycone is neutral sugar.

Composition of neutral sugar [b]	(mol %)
Glucose	25.1
Galactose	43.1
Mannose	21.9
Arabinose	9.9
Fucose	0

Neutral sugar contains glucose, galactose, mannose, and arabinose, but does not contain fucose.

a) Uronic acid was determined by carbazole method, and neutral sugar by phenol sulfate method.

b) The composition of neutral sugar was analyzed by gas chromatography after transforming methyl glycoside produced by methanolysis into trimethyl.

[Analysis of aglycone]

Molecular weight: 4000±2000 by Toyopearl HW-40F gel filtration method.

Analysis of infrared absorption (IR)

IR was measured by using KBr disk.

As a result, an absorption having a peak at 3400 cm^{-1} was detected in a range of 3500 to 3700 cm^{-1}. This absorption indicates the presence of phenolic hydroxyl group. The absorption around 1600 cm^{-1} shows an aromatic double bond. Since there is no absorption of carbonyl group around 1700 cm^{-1}, there is no ester bond as noted in tannin, and it is proved to be a compound polymerized by ether bond as observed in lignin.

Including the fingerprint region, the entire spectrum is extremely similar to that of lignin (alkali).

From the above results of IR spectrum, the aglycone of this glycoside is estimated to be a lignin-like compound, not tannin-like compound.

Analysis of ultraviolet absorption (UV)

Maximum absorption was detected at 280 nm, and minimum absorption at 260 nm.

280/260 = 1.02

Lignin also has a similar UV spectrum.

280/260 = 1.03

The presence of aromatic group (probably phenol) is indicated by UV spectrum.

Analysis of electron spin resonance (ESR)

Same as in lignin, g = 2.004 ESR signal is detected, and it is known to contain a structure having a stable free radical. Such signal is not observed in tannin.

[Analysis of lignin glycoside]

The feature as the entire lignin glycoside is as follows.

Element analysis	(weight %)
C	40.38
H	4.85
O	54.74
N	0.03 or less
S	0

Since nitrogen and sulfur are not contained, it is free from protein and sulfate group, and the molecular weight by Sepharose CL-4B gel filtration method about 9000.

The bonding ratio of lignin and sugar is about 1.5: 1 by weight.

Therefore, it is a feature of this glycoside that it contains neutral sugar by about 85% of the total weight as sugar component (glycone). The neutral sugar includes glucose, galactose, mannose, and arabinose. The non-sugar component (aglycone) is composed of lignin.

This glycoside is an O-glycoside having the reduction end lactol hydroxyl group of sugar bonded in ether form with

alcoholic or phenolic hydroxyl group of lignin by dehydration and condensation. The bonding ratio of lignin and sugar is about 1.5:1 by weight, and in particular it is a lignin glycoside (as classified by the feature of aglycone) with molecular weight of about 9000 with a particularly large content of neutral sugars.

The estimated structural model of the glycoside is as shown in the diagram.

Test example 1

Inhibitory effect on poly-(ADP-ribose) glycohydrolase

To a buffer for assay (0.01% bovine serum albumin, 10 mM mercaptoethanol, 50 mM potassium phosphate, pH 7.0), ^{3}H-$(ADP\text{-}ribose)_{n=15}$ was added, and to 27 µl thereof, further, the substance to be tested and nuclear derivative poly-(ADP-ribose) glycohydrolase solution prepared from human placenta were added to make up 30 µl in total, which was incubated for 1 hour at 37°C. Later, the reaction solution was absorbed in DE81 filter paper, and the filter paper was washed in water, ethanol and acetone, and was dried, and the unreacted substrate ^{3}H-(ADP-ribose) was measured by liquid scintillation counter, and the inhibitory action of the test substance on this enzyme was investigated. Results are shown in Table 1, which shows all tested substances inhibited poly-(ADP-ribose) glycohydrolase dose-dependently.

Table 1

Inhibitory activity of lignin glycoside on poly-(ADP-ribose) glycohydrolase

Concentration of lignin glycoside (µg/ml)	Activity of poly-(ADP-ribose)glycohydrolase (%)
0	100
0.3	86
1.0	24
3.0	4

Test example 2

Inhibitory effect on gene expression

The gene expression system used in the test was 341 strains of mouse mammary tumor cells having mouse mammary tumor virus (MMTV) genes which are saccharide corticoid susceptible genes. The cell, in the presence of saccharide corticoid, expresses 35S RNA, and 24S RNA further undergoing splicing. This expression can be detected by using cDNA of the env portion. Herein, the test substance was added to 341 strains by 30 μg/ml, and incubated for 30 minutes at 37°C, and dexamethazone was added to the system by 10^{-7}M, and it was further incubated for 1 hour. Then, 341 cells were collected, high molecular RNA was extracted by guanidine-hydrochloric acid method, and after treating for 5 minutes at 60°C (20 mM MDPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA), electrophoresis was conducted (40 V, 16 h) by 1.2% agarose gel (same buffer). Consequently, transferring to nitrocellulose, ^{32}P-MMTV-DNA (cDNA specific to env) was hybridized, and an autoradiogram was prepared by X-ray film. Using the autoradiogram, the concentration of 35S and 24S RNA bands was measured by densitometer, and the RNA expression amount was determined, and the result was compared with the control without addition of test substance, and the RNA expression inhibitory rate was calculated. As the result is shown in Table 2, the test substance presented the MMTV gene expression inhibitory action.

Table 2

Action of lignin glycoside on expression of mouse mammary tumor virus (MMTV) gene

	Dexamethazone (10^{-7}M)	Lignin glycoside (30 μ g/ml)	Sensitivity of 35S, 24S bands
1	+	+	++++
2	-	-	+
3	-	-	+
4	+	+	++

1: positive control, 2and3: negative control, 4: test group

Test example 3

Carcinostatic effect on mouse experimental tumor

In the abdominal cavity of mouse, 1×10^6 tumor cells of sarcoma 180 were transplanted, and the test substance was continuously administered intraperitoneally for 1 to 4 days after transplantation. The antitumor activity was determined by the survival rate by comparison with the normal saline administration group.

The experiment was terminated on the 45th day after tumor transplantation. The results are shown in Table 3, and the test substance presented a carcinostatic action.

Table 3

Carcinostatic action of lignin glycoside on mouse tumor sarcoma 180

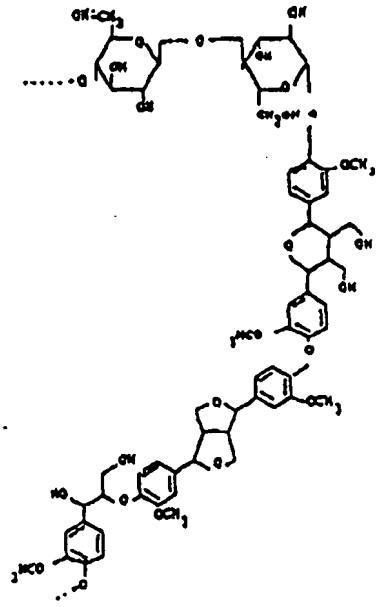
Sample	Dose (mg/kg)	T/C (%)
None		100
Lignin glycoside	40×4	135
	20×4	177
	10×4	157
	5×4	122

The lignin glycoside was administered for 4 days consecutively from the day after transplantation.

The drawing shows the estimated structure of the lignin glycoside of the invention.

Applicant: The Green Cross Corp.

Attorney: Hajime Takashima, patent attorney



Procedure Amendment

November 20, 1990

To: Secretary-General, Patent Office of Japan

1. Indication of the case
   Patent Application No. 113049 (1990)
2. Title of the invention
   Lignin glycoside and its use
3. The amending party
   Relation with the case: Applicant
   Name: The Green Cross Corp.
4. Attorney
   Address: Yuki Bldg., 3-3-9 Hiranomachi, Chuoku, Osaka 541
   Tel. 06-227-1156
   Takashima International Patent Office
   Name: Hajime Takashima, patent attorney (8079)
5. Date of amendment command
   July 31, 1990 (mailing date)
6. Object of amendment
   The line of "Brief description of the drawing" of the specification.
7. Content of amendment
   (1) To replace page 19 of the specification with the attached sheet. (There is no change in the content except for the description of the amendment.)

The experiment was terminated on the 45th days after tumor tumor transplantation. The results are shown in Table 3, and the test substance presented a carcinostatic action.

Table 3

Carcinostatic action of lignin glycoside on mouse tumor sarcoma 180

Sample	Dose (mg/kg)	T/C (%)
None		100
Lignin glycoside	40×4	135
	20×4	177
	10×4	157
	5×4	122

The lignin glycoside was administered for 4 days consecutively from the day after transplantation.

4. Brief Description of the Drawing

The drawing shows the estimated structure of the lignin glycoside of the invention.

Applicant: The Green Cross Corp.
Attorney: Hajime Takashima, patent attorney